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SPERM CHEMOTAXIS IN *OIKOPLEURA DIOICA* FOL, 1872 (UROCHORDATA: LARVACEA)

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ABSTRACT

An alcohol extract of unfertilized eggs of the larvacean, *Oikopleura dioica,* can attract sperm over a distance of at least 80 μm from an artificial source. The sperm, which normally swim in wide circles or straight lines, alter their path to form small loops between straight or slightly curved segments directed up the gradient. During the first loop, the velocity of sperm increases 50%. The new velocity is maintained as long as the cells are influenced by the attractant. Once sperm reach the center of the gradient, the path alters to the form of enlarging concentric circles which eventually attain the diameter of the circles made in sea water. *O. dioica* sperm and sperm attractant are species-specific in tests against attractants and sperm of sessile tunicates. It has not yet been possible to test the species-specificity against other larvaceans. We estimate that sperm chemotaxis in *O. dioica* increases the chance of sperm-egg collisions from 4 to 15 times. This is mainly due to an increase in apparent diameter of the egg and also to an increase in the velocity of attracted sperm. Rapid population increase is characteristic of *O. dioica* under appropriate conditions. An increase in the probability of fertilization produced by sperm chemotaxis may be an additional factor leading to decreased generation time for the population as a whole.

INTRODUCTION

Larvaceans are adult planktonic urochordates which resemble the tadpole larva of sessile urochordates (Tunicata). They are widely distributed in tropical and temperate oceans (*e.g.,* Forneris, 1957; Fenaux, 1967) and may be found in immense numbers under certain circumstances (Seki, 1973; Wyatt, 1973). Larvaceans may rapidly attain large population size because they take advantage of short term conditions optimal for maximum growth of the population. They possess very rapid development (Galt, 1972; Fenaux, 1976) coupled with rapid growth to sexual maturation (Fenaux, 1976; Paffenhöfer, 1976). Generation times of 10 days or less have been measured in enclosed water columns (King *et al.,* 1980; King, 1982).

In contrast to other larvaceans, *Oikopleura dioica* is dioecious. Spawning is random and may be triggered by physical means, such as turbulence or contact with another object (Galt, 1972). The completely transparent eggs are denser than sea water and sink after spawning (Bienfang and King, unpub.). Little is known about gamete interactions in these organisms. If it is advantageous to decrease development time in order to react quickly to favorable environmental conditions, then shortening

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the interval between the spawning act and the time of fertilization may be important in situations where the presence of the opposite sex cannot be predicted. One method for ensuring fertilization and decreasing the time that the eggs remain unfertilized is sperm chemotaxis where sperm move closer to the egg from some distance away by following a gradient of a substance released by the egg (Miller, 1973). In taxa where sperm chemotaxis has been described (Miller, 1966; 1975; 1977; 1979a), it has been noted that the attractant often increases sperm velocity, further decreasing the time of sperm approach. Sperm of many planktonic hydromedusae (Miller, 1979a, b) and sessile tunicates (Miller, 1975; 1982) exhibit chemotaxis. Here we describe this phenomenon in a planktonic urochordate, and speculate on its possible impact on the population dynamics of *O. dioica*.

Materials and Methods

Sexual specimens of *Oikopleura dioica* were gently removed from the ocean with a large bore pipette as they drifted past the dock at the Friday Harbor Laboratories. The animals were made visible using a submerged night-light (Woodland, Inc.). Ripe males could be distinguished by the swollen brilliant white testes. The ovaries of females were also swollen and white but somewhat less brilliant. Individuals were kept segregated by sex in small clean finger bowls and used immediately after collection.

Gametes were obtained by pricking the gonad surface of individuals which had been previously transferred through several changes of HA-Millipore (0.45 μm) filtered sea water to remove supernumerary sperm. Eggs were permitted to settle and the sea water removed. The damp eggs were extracted for 10–20 minutes in 95% ethanol to yield the active extract (Miller, 1979a). Aliquots of this were air-dried, diluted into an equivalent volume of sea water and injected into a suspension of actively moving sperm. The sperm suspension was placed on a standard microscope slide within a 2.4 cm^2 area previously covered with a thin layer of 1% bovine serum albumin in distilled water, to create a flat puddle a few mm deep. The egg extract was injected with an RGI micrometer syringe connected by thin polyethylene tubing to a micropipette of 30 μm tip diameter. Back pressure was controlled by filling the tubing and syringe with mineral oil. The pipette was lowered into the puddle and brought to the slide surface while under observation in dark-field illumination with a 10× objective.

Like other invertebrate sperm, *O. dioica* sperm become thigmotactic on non-sticky, smooth surfaces. This allows the objective to be focussed on the thigmotactic cells, which remain on the microscope slide surface indefinitely. The rationale for the use of thigmotactic sperm for observation of chemotactic behavior and the probable artifacts inherent in this approach are discussed in Miller (1973). Sperm behavior was observed and photographed at 12 fps, with 4× reversal film using a Bolex 16 mm camera. The developed film was analyzed with a Kodak "Analyst" projector by projecting the film onto tracing paper and plotting the path of the sperm cells by hand. Sperm velocity was determined by measuring the distance the sperm head traveled each frame.

We tested for species-specificity by confronting the sperm of *O. dioica* with egg extracts from several sessile tunicates, and the various tunicate egg extracts with the sperm of the larvaceans and tunicates. The numerical estimate of extract activity used in this work is the titer, or the number of serial half-dilutions required for complete loss of activity against homo- or heterospecific sperm (Miller, 1979a).

RESULTS

Under the standard conditions of observation, *Oikopleura dioica* sperm swimming in sea water make relatively straight (Fig. 1A) or circular paths (Fig. 1B, 2). The average velocity during these "control" trails is 75.6 μm/s (Table I). Infrequent,

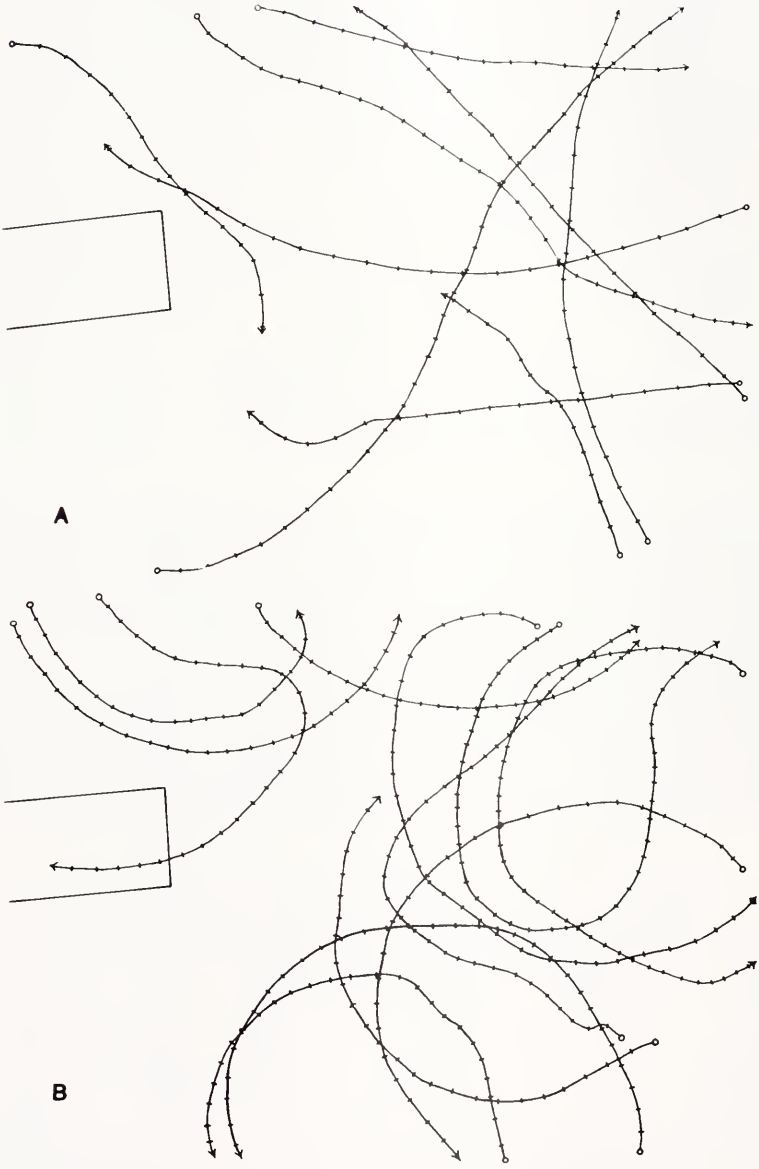

FIGURE 1. Paths of *Oikopleura dioica* sperm in the presence of a pipette injecting sea water. A. Mainly straight or slightly curved trails. B. Mainly curved trails. Pipette diameter is 30 μm. Each interval on the trail represents 0.08 s.

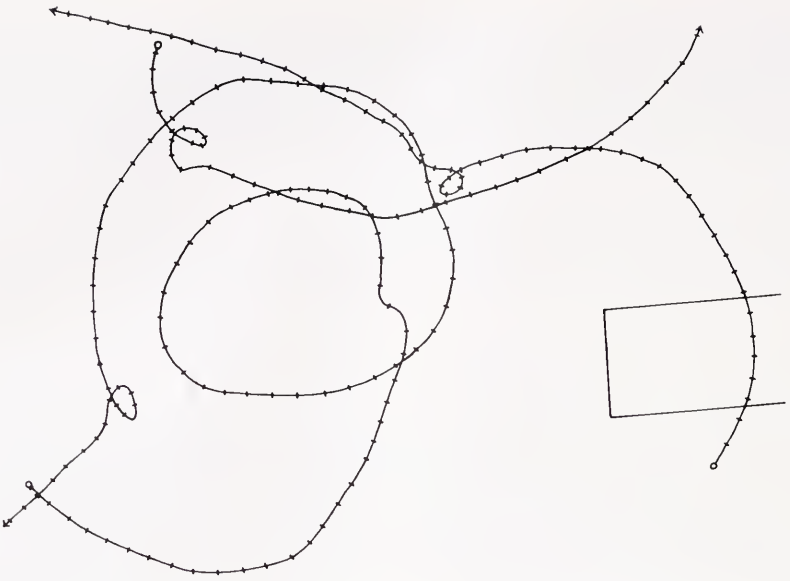

FIGURE 2. Paths of *Oikopleura dioica* sperm in the presence of a pipette injecting sea water. Curved trails with rare, random loops. Pipette diameter is 30 μm.

random turns in the form of sharp loops may occur in some trails within 4–5 frames (approximately 0.35 s) (Fig. 2). The direction taken after these loops have been completed is roughly 270° relative to the original path direction. The form of the new path is the same as the original. Injection of sea water into the sperm suspension produces no change in sperm motility or direction as long as the rate of injection is slow enough to prevent physical shifting of the sperm.

If a sea water solution of *Oikopleura* egg extract is injected (experimental trails), the sperm behave quite differently (Fig. 3). Sperm enter the field on a typical preattraction circular path at about average velocity (72.0 μm/s; Table I) but, about 130 μm away from the pipette tip, undergo a looping behavior which brings them closer to the pipette tip. The average velocity during these trails is 96.2 μm/s (Table I).

TABLE I

Average velocities along Oikopleura *sperm trails before and during chemotaxis*

Trail type	Number of trails	Number of measurements	Mean (μ/s)	SD	SE	*P*
Control trails	21	852	75.61	1.896	±.065	<.001[a]
Experimental trails	11	681	96.22	2.502	±.096	
Pre-attraction	11	148	71.97	1.565	±.129	<.001[b]
Post-attraction		148	109.88	1.652	±.136	

Control and *Experimental* trails refer to groups of trails in sea water and exposed to a gradient of sperm attractant, respectively. *Pre-attraction* and *Post-attraction* refers to measurements made at the start of the 11 experimental trails and the same number of measurements made at the end of the same set of trails, respectively.

a, *t*-test, groups; control trails *versus* experimental trails.

b, *t*-test, pairs; pre-attraction *versus* post-attraction in experimental trails.

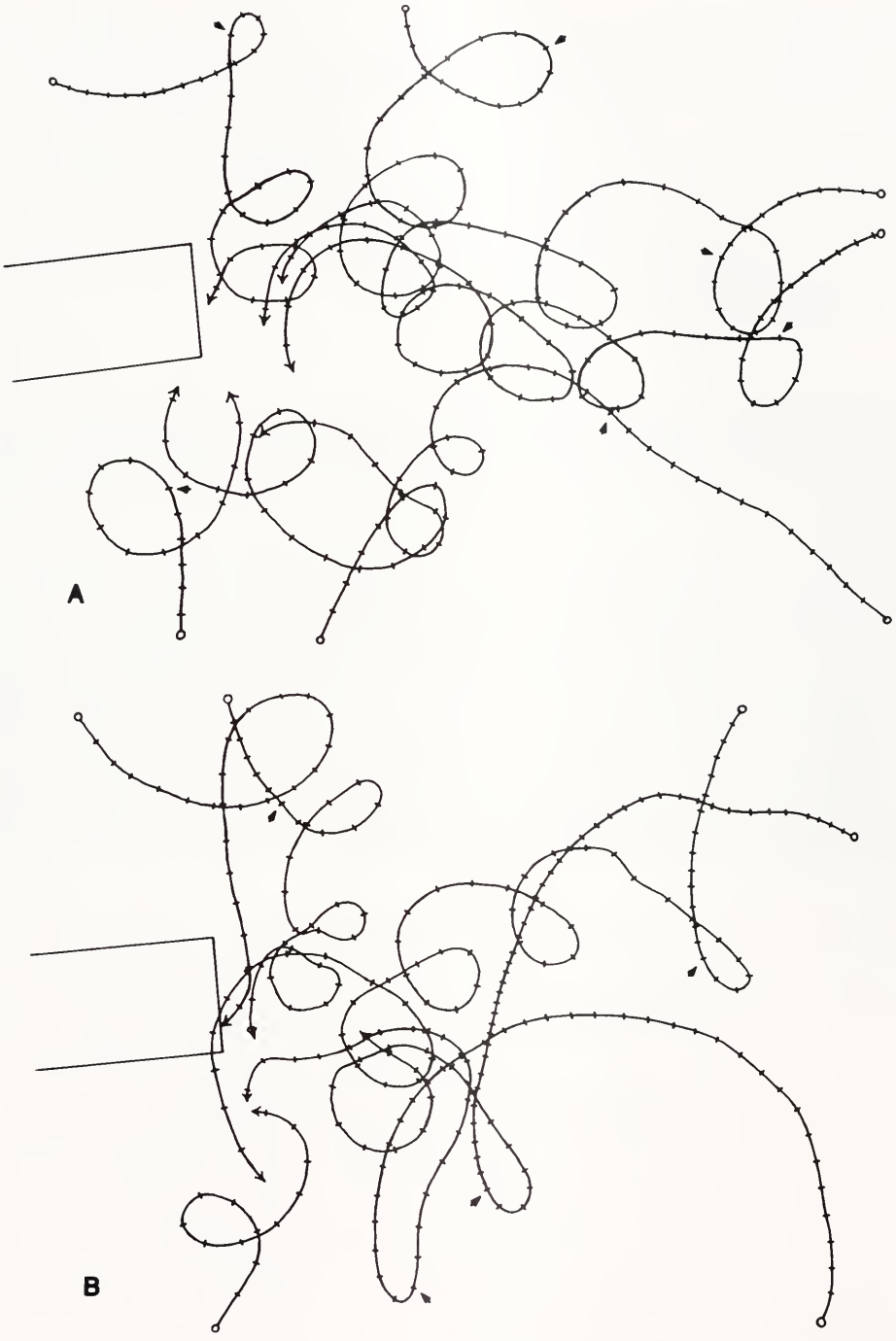

FIGURE 3. Paths of *Oikopleura dioica* sperm in the presence of a pipette injecting *O. dioica* egg extract with a titer of 9–10. Trails in A and B were obtained from an 18.7 s film sequence and trail positions have been slightly adjusted for the best demonstration of their characteristics. Arrows indicate point of acceleration of sperm in response to the attraction gradient. Pipette diameter is 30 μm.

TABLE II

Oikopleura *sperm trail loop and circle characteristics before and during sperm chemotaxis*

	Loops[0]		Circles[0]	
	Diameter (a, b)	Length (l)	Diameter (d)	Circumference (C = d)
	$a \times b$			
Before	9.4 × 3.4 μm (5)*	24.7 ± 1.14 μm (5)	97.7 ± 5.8 μm (20)	306.8 ± 15.9 μm (20)
During	24.2 × 17.9 μm (25)	66.5 ± 4.4 μm (25)	42.4 ± 5.5 μm (9)	133.3 ± 17.4 μm (9)

* number of measurements.
[0] refer to Figure 4.

Table II and Figure 4 present measurements of the loops and circles made in control and experimental trails. The average loop is 2.7 times longer and 5 times broader in the experimental (attraction) trails than during the control trails. During the first looping maneuver, the velocity of the sperm increases significantly (paired *t*-test; $P < 0.001$) (Table I) and the new speed (109.9 μm/s) is maintained for the rest of the trail.

Once the attracted sperm arrive at the pipette tip, they begin to circle around it (Fig. 5, 6A). The circles of all the sperm become more or less concentric, with an average diameter half of those made during normal swimming (Table II; compare Figs. 1A, B with Figs. 5, 6A). The concentric circular paths enlarge in diameter, resembling those seen prior to attraction (Fig. 5A, 6B). All sperm swim counterclockwise during this behavior as they did in the circles and loops made before attraction. Their velocity remains high (109.9 μm/s). The cells seem to have entered a new, stable motility configuration and behave as though the attraction gradient is no longer present. The result of this sequence of behaviors is a rapid shift of the sperm population toward the pipette tip. By the end of the film sequence, few sperm are found at the margins of the area of observation.

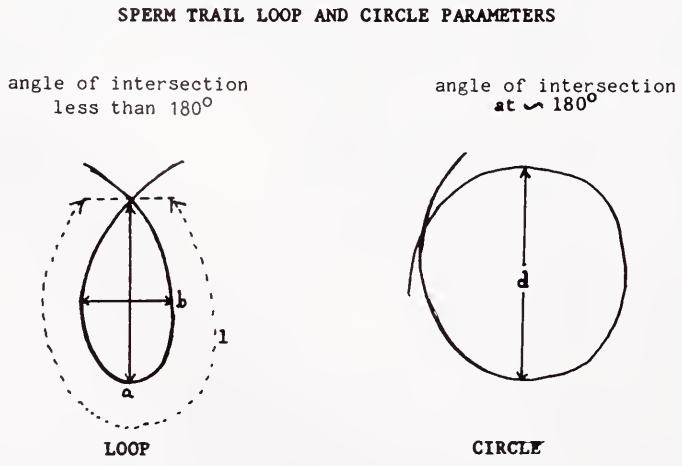

FIGURE 4. Diagrammatic representation of sperm trail loops and circles, with measurement parameters used to determine loop and circle sizes.

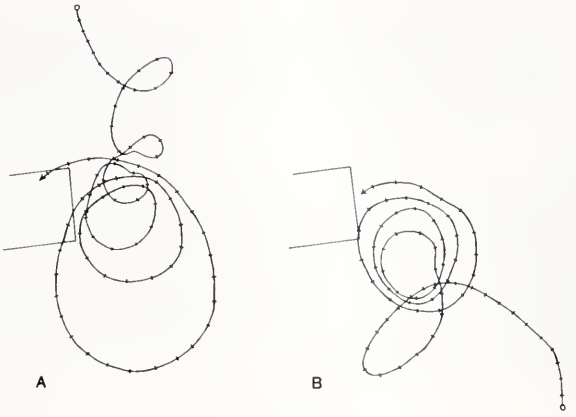

FIGURE 5. Two trails of attracted sperm showing the start of the concentric circling behavior that is the result of sperm chemotaxis. 5A shows the characteristic progressive enlargement of the circles. Pipette diameter is 30 μm.

It is evident that the sperm are directed toward the pipette tip when the *O. dioica* egg extract is released. To confirm this, the pipette was moved about 0.15 mm from the outer margin of the old aggregation and a new injection made. The sperm move from the old aggregation into the new injection area, where a new swarm is formed of sperm swimming concentrically about the pipette tip. Therefore, not only is a gradient of attractant required for sperm aggregation, but the same cells can be re-attracted by the same egg extract.

In three cases we were able to follow the movement of very small particles (~1 μm in diameter) in front of the pipette as the attractant was injected. Each of these cases differed in the force of the injection. In the first, enough force was exerted to push the particles 90 μm away from the tip before they came to rest. Sperm were

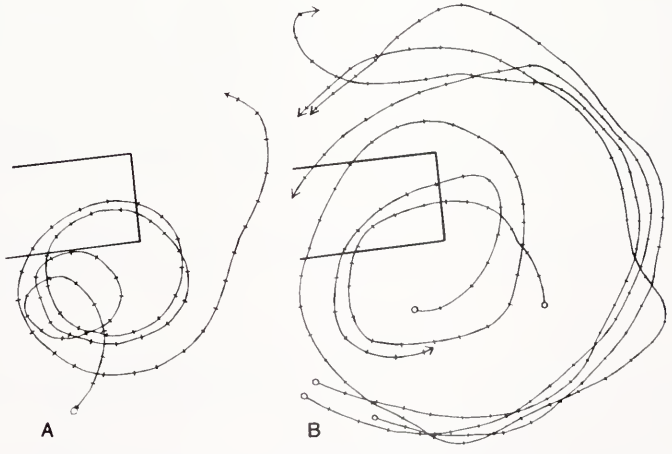

FIGURE 6. A. Another trail showing the transition to concentric circling behavior and the transition from small to large circles. B. A portion of a plot of sperm circling around the pipette tip at the end of the preparation (injection stopped). This is 5.3 s of a film sequence showing this behavior only.

seen to respond 40 μm further away from the tip. In the second case, the particles came to rest 60 μm away from the pipette tip and the sperm were seen to turn a further 40 μm away. In the third case, no particle movement occurred. In this case, the sperm turned 80 μm away from the pipette tip. These three cases suggest that sperm can respond to attractant which has diffused at least 40 to 80 μm beyond the area of injection.

Tests of the effects of egg extracts from sessile tunicates on *O. dioica* sperm have yielded complete species-specificities in all cases. The active egg extracts of *Ascidia callosa* (titer = 11) and *Chelyosoma productum* (titer = 5) do not attract *O. dioica* sperm, whereas the behavior of the sperm of *Corella inflata, Corella willmeriana, Ciona intestinalis, Ascidia callosa, Chelyosoma productum, Styela montereyensis, Styela gibbsi,* and *Halocynthia igaboja* remains unaffected by the presence of a gradient of *O. dioica* egg extract (titer = 8–9).

Discussion

We have demonstrated that *O. dioica* spermatozoa, when confronted with a gradient of an egg extract, are capable of sperm chemotaxis. The trails of attracted sperm strongly resemble those of chemotactic sperm of other invertebrates (Miller, 1966; 1975; 1977; 1979a). They most particularly resemble chiton sperm trails (Miller, 1977) and those of asteroid and holothuroid sperm (Miller, 1981; in prep.). Sperm chemotactic behavior is reversible and can be highly species-specific (Miller, 1979a). Recent work has shown considerable specificity at the genus level in the ascidians (Miller, 1982). Species-specificity between the larvaceans and the ascidians is therefore to be expected, and evidence for it has been presented. Interspecific comparison of sperm chemotaxis between two species of larvaceans has not been possible for lack of suitable material.

When *Oikopleura* sperm chemotactic behavior is initiated, sperm velocity increases 50% and remains at this level for the rest of the trail. Velocity increase has been observed during cnidarian sperm chemotaxis (Miller, 1966). In contrast to chiton, cnidarian, and *Oikopleura* sperm, the sperm of the sessile tunicates *Ciona* and *Styela* and those of several echinoderms show no velocity increase during chemotactic turning or subsequent movement up the gradient (Miller, 1975; 1981; 1982; in prep.). No further changes in velocity occur during subsequent reorientations of larvacean sperm, suggesting that reorientation behavior and velocity increase may be independent in *O. dioica* sperm, unlike chiton sperm, where small velocity adjustments occur during every reorientation loop (Miller, 1977). The source of the activation stimulus may be the sperm attractant itself, though it is possible that the egg extracts also contain a motility activator (Hansbrough and Garbers, 1981).

Unlike sessile tunicates, larvacean populations are not limited by availability of settling substrate for the larvae (Grosberg, 1981), but rather by food supply and predation (King, 1982). The ability of larvaceans to rapidly increase population size under certain conditions has been documented (King *et al.,* 1980; King, 1982). Quantitatively, the relative magnitude of the factors which aid in this increase are uncertain. Any factor which shortens the developmental time from spawning to sexual maturation would be of importance, particularly in this instance, where sexual aggregation may not occur prior to spawning.

Larvacean eggs have a density greater than sea water and sink at about 25 m/day (300 μm/s) (Bienfang and King, unpub.). The sperm velocity is moderate

(~80–110 μm/s) and the sperm is quite small (~20 μm in length; Flood and Afzelius, 1978), though large numbers are shed by each ripe male. Spawning is cataclysmic and asynchronous in both sexes (Galt, 1972) so there should not be a uniform concentration of sperm or eggs in the water column. Both gametes have a fertilizable life of about 24 h (Galt, 1972).

Assuming the sperm are swimming in random directions relative to the egg, the chances of an egg being fertilized at a particular time depend directly on local sperm concentration, average swimming speed of sperm, the average age of the sperm and eggs, the egg surface area and its sinking rate (Rothschild and Swann, 1949; 1951). However, in larvaceans, some of these factors are not constant. Sperm velocity increases and sperm path direction is determined by the presence of eggs, once the sperm arrive within a minimum distance of 80 μm from the egg. The effect of this is to increase the chances of a nearby sperm making contact with the egg surface by enlarging the effective egg diameter from 80 μm (Delsman, 1912; Galt, 1972) to 240 μm or more.

The number of collisions of sperm with a non-sinking egg per unit time (Z) is a function of the number of sperm (n), their average velocity (c), and the square of the egg radius (r): ($Z = \pi r^2 nc$; Rothschild and Swann, 1951). Increasing velocity by 50% (from 70 to 110 μm/s) will increase the number of collisions by two-thirds. Increasing radius of the egg by three (from 40 to 120 μm) increases collision rate 9 times. The estimated increase in collisions due to sperm chemotaxis compared to the "standard" fertilization paradigm is approximately 15 times for *O. dioica,* assuming no increase in sperm numbers.

Unfortunately, sperm cannot swim as fast as eggs can sink. However, both sperm and eggs occur in an aqueous medium under a low Reynolds number regime where viscosity effects are dominant, flow is laminar, and nearby water tends to move with objects that are subject to an external force (Purcell, 1977; Koehl and Strickler, 1981). In such a situation the sinking egg (Reynolds number = 0.02) will have a layer of hydrodynamically constrained water (a boundary layer) associated with it. As the egg sinks, water at and beyond the boundary layer reaches a velocity, relative to the egg, equivalent to the egg sinking rate. Within the boundary layer, a velocity gradient exists such that, at 20 μm away from the surface of the egg, water velocity relative to the egg and maximum measured sperm swimming speed are equivalent (White, 1974). Assuming that sperm attractant is continually released into the boundary layer, the increase in effective egg diameter by 50% (beyond 20 μm the sperm cannot catch the sinking egg) and the concomitant increase in sperm swimming speed upon contact with attractant, yields about a four-fold increase in successful sperm egg collisions. This is probably a worst case estimate of the efficacy of sperm and sperm attractant interactions for larvaceans in the pelagial. *Oikopleura dioica* is usually most abundant in the surface mixed layer. Here, small scale turbulence in the water column may provide long term residency in the mixed layer for sperm and egg by effectively altering egg sinking rates.

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